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MASP-3 and Its Association with Distinct Complexes of the Mannan-Binding Lectin Complement Activation Pathway

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Summary

The mannan-binding lectin (MBL) pathway of complement activation is part of the innate immune defense. The binding of MBL to microbial carbohydrates activates the MBL-associated serine proteases (MASPs), which recruit the complement factors, C4 and C2, to generate the C3 convertase or directly activate C3. We present a phylogenetically highly conserved member of the MBL complex, MASP-3, which is generated through alternative splicing of the MASP-1/3 gene. The designation of MASP-3 as a protease is based on homology to known MASPs. Different MBL oligomers were found to have distinct MASP composition and biological activities. MASP-1, MAp19, and direct C3-cleaving activity are associated with smaller oligomers whereas MASP-3 is found together with MASP-2 on larger oligomers. MASP-3 downregulate the C4 and C2 cleaving activity of MASP-2.

Introduction

The host defense against microorganisms relies on both innate and adaptive elements (Hoffmann et al., 1999; Medzhitov and Janeway, 2000). Innate immunity offers the main resistance to a microbial pathogen within the first minutes, hours, or days of an infection. However, it has lately transpired that the innate immune system also plays a significant role for ensuring the adaptive immune response (Dempsey et al., 1996; Fearon and Locksley, 1996). The innate immune system is activated as soon as pathogens or enviromental antigens are flagged by soluble or membrane-bound recognition molecules. These are germline encoded proteins and have as such evolved through natural selection to efficiently detect patterns (pathogen-associated molecular patterns [PAMPs] [Janeway, 1989]) that are evolutionarily conserved on microorganisms. This contrasts to the adaptive immune system where the recognition molecules (antibodies and T cell antigen receptors) achieve their diversity through ontogenic rearrangement of the genes.

One plasma protein, which enables the innate immune system to distinguish self from non-self, is mannan-binding lectin (MBL), a member of the complement system. The complement system comprises a series of plasma and cell membrane proteins (Law and Reid, 1995). It represents a well-known antimicrobial defense mechanism of major clinical importance (Walport, 2001a, 2001b) but also serves as a natural adjuvant, enhancing and directing the adaptive immune response (Carroll, 2000). Thus, animals deficient in complement factor C3 or C4, either acquired (Pepys, 1974) or genetically as a result of disruption of the genes by homologous recombination, have an impaired humoral response to T cell-dependent antigens associated with reduced number and size of germinal centers and impaired retention of antigen on folicular dendritic cells (Fischer et al., 1996). This results from defaults in the interaction between the fragments of complement factor C3 (C3b and/or C3d) and C4 (C4b) attached to the antigen and receptors for these fragments (Fearon and Carroll, 2000).

The initiation of the complement cascade in the early phase of an immune response may be achieved through three pathways. (1) Natural polyreactive IgM antibodies, secreted by B-1 cells, can lead to activation of complement via the classical pathway. (2) The deposition of fragments of C3 on certain structures that impede the control of the alternative pathway will initiate this pathway. (3) The third pathway of complement activation, the mannan-binding lectin (MBL) pathway, also termed the lectin pathway, was recently discovered. This pathway is initiated on surfaces with a carbohydrate decoration not normally found in the body.

Deficiency of MBL is emerging as the most common human immune defect (Turner and Hamvas, 2000). The level of MBL is largely genetically determined with mutations in the coding region as well as in the promoter region, causing low concentrations (Lipscombe et al., 1996; Madsen et al., 1995). MBL has the ability to bind to carbohydrate structures on the surface of microorganisms (including bacteria, viruses, and fungi) and mediate the deposition of complement factors (Ikeda et al., 1987; Matsushita and Fujita, 1992; Ji et al., 1993; Thiel et al., 1997). This promotes killing of the microorganism via the membrane attack complex, as well as through enhanced phagocytosis of the microorganism due to the opsonizing effect of deposited C3b. MBL may also act as an opsonin itself (Kuhlman et al., 1989) through interaction with phagocytic receptors (Ghiran et al., 2000). Interestingly, a recent report suggests involvement of MBL in the class switch of antibody production toward MBL-reactive carbohydrate epitopes (Selander et al., 1999).

The biological significance of MBL was realized when MBL deficiency was found to be the likely cause of opsonin deficiency in children with an unexplained pro-

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pensity for frequent, often serious infections (Super et al., 1989), an observation subsequently supported by numerous epidemiological investigations (Summerfield et al., 1997; Garred et al., 1999).

MBL belongs to the collectin family of proteins and is an oligomer of structural subunits each composed of three identical 30 k M_r polypeptides with a C-type carbohydrate recognition domain joined to a collagenous region (Turner, 1996). The overall structure is thus similar to that of C1q, the antibody-recognizing moiety of the first component of the classical complement activation pathway.

The activation via MBL was found to proceed via specific MBL-associated serine proteases (MASPs) of a modular structure identical to that of C1r and C1s, i.e., an A chain composed of CUB1, EGF, CUB2, CCP1, and CCP2, joined by a linker to the B chain encompassing the serine protease domain (Matsushita and Fujita, 1992; Thiel et al., 1997). The MBL complex has been reported to contain MASP-1, MASP-2, and a smaller nonenzymatic component, MAp19 (Stover et al., 1999) or sMAP (Takahashi et al., 1999). The stoichiometry of the MBL/MASP complex is unknown, and it appears that the MASPs do not function in concert as do C1r and C1s; rather MASP-1 shows the ability to activate C3 (Matsushita and Fujita, 1992, 1995a; Matsushita et al., 2000) while MASP-2 activates C4 and C2 (Thiel et al., 1997; Matsushita et al., 2000; Vorup-Jensen et al., 2000). We report here on the discovery of a third MASP. We describe its association with distinct MBL complexes and discuss evolutionary aspects of MASP-3.

Results

Identification of a MASP

Analysis by SDS-PAGE of plasma proteins, purified through calcium-dependent binding to matrices derivatized with GlcNAc, revealed an unidentified protein of 42 k M_r. The N-terminal sequence of the protein suggested that it represented the start of a serine protease domain. This sequence, as well as those of some trypsin-generated peptides, is shown underlined in the subsequently determined complete sequence of the protein (see below).

Antibody was raised against a synthetic 19 amino acid residue peptide representing the N-terminal sequence. Two-dimensional SDS-PAGE and Western blotting of a lectin preparation using this antibody as a probe revealed that the presumed serine protease domain of 42 k M_r was derived from a protein of $M_r \approx 105$ k M_r. Before activation, the 105 k M_r protein forms a disulphide-linked dimer (Figure 1A). Activation splits the 105 k M_r protein into 42 k M_r and 58 k M_r chains. The longer chain is not seen in these Western blots, as the antibody used does not detect it. The structure thus resembles the A and B chain structure of other serine proteases with the shorter B chain encompassing the serine protease domain.

Association of the Serine Protease with MBL

Analytical affinity procedures showed that the protein occurred in plasma complexed with MBL (Figure 1B). The protein was thus bound to solid-phase anti-MBL antibody when MBL-sufficient serum was applied, but not when MBL-deficient serum was applied. When MBL was added to MBL-deficient serum, the protein was again bound to the solid phase (Figure 1B, lanes 3 and 5). The protein was accordingly termed MBL-associated serine protease-3 (MASP-3).

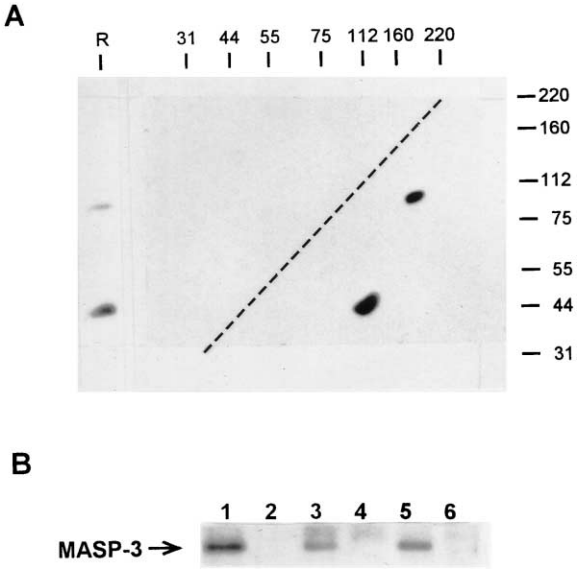

Figure 1. Characterization of MASP-3 and Its Association with MBL

(A) Two-dimensional SDS-PAGE Western blot of MBL complexes purified by affinity chromatography on mannan-Sepharose. The first dimension (horizontal) was run under nonreducing conditions. The lane was then reduced and run in the second dimension. The gel was blotted and developed with antibody against the N-terminal of the 42 k M_r protein. The second dimension gel was prepared with a separate well for a reduced sample of MBL complexes (lane R), which thus illustrates the pattern after standard one-dimensional electrophoresis. The positions of the M_r markers are indicated.

(B) Association of MASP-3 with MBL. Samples (100 μl) of sera diluted with an equal volume of TBS were incubated in microtiter wells coated with monoclonal anti-MBL antibody, eluted with 100 μl SDS sample buffer for 10 identical wells (Thiel et al., 2000), and examined by SDS-PAGE Western blotting using antibody against the N-terminal of the 42 k M_r protein. The samples were: 1, normal serum containing MBL 2 μg/ml; 2, purified MBL (Vorup-Jensen et al., 2000) (1 μg); 4 and 6, two MBL-deficient sera (MBL concentrations $<$ 20 ng/ml); 3 and 5, the same two MBL-deficient sera with MBL added to 2 μg/ml.

Association of MASP-3 with Distinct Subsets of MBL Complexes

MBL complexes could be separated into different structural and functional forms by ion-exchange chromatography as well as by sucrose gradient centrifugation. Four distinct MBL bands, MBL-I, II, III, and IV, were revealed by nonreducing SDS-PAGE, with mobilities corresponding to approximate M_rs of 275 k M_r, 345 k M_r, 580 k M_r,and 900 k M_r (Figure 2B). On ion-exchange chromatography, they were eluted in that order by increasing salt concentration, and on sucrose gradient centrifugation they showed increasing sedimentation rates in the same order (Figures 2A, 2B, and 2G). Following both fractionation procedures, MASP-1 and MAp19 were found to be associated largely with MBL-I, and MASP-2 and MASP-3 largely with MBL-II. The ability to activate C4, the first

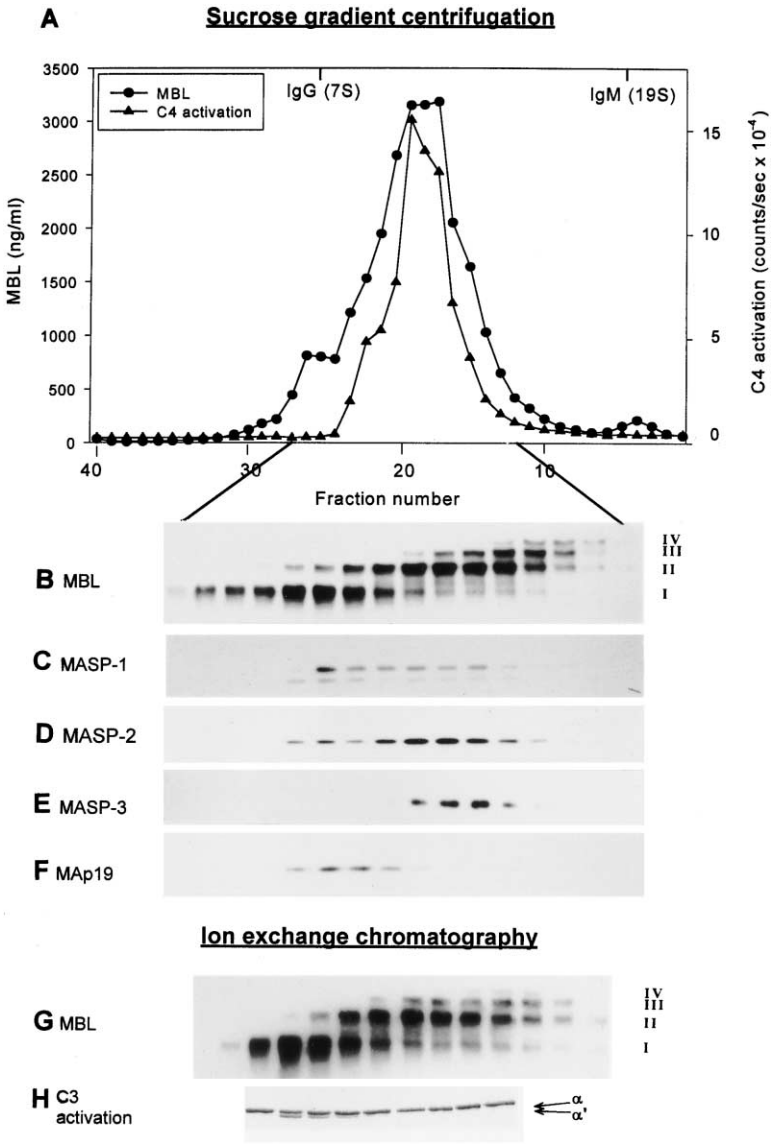

Figure 2. MBL Complexes of Different Sizes and Compositions

(A) Sucrose gradient centrifugation showing the C4 activating capacity and the MBL content of the fractions. The positions of 7 S IgG and 19 S IgM are indicated. (B) SDS-PAGE Western blot of the fractions developed with anti-MBL antibody, (C) with anti-MASP-1 antibody, (D) with anti-MASP-2 antibody, (E) with anti-MASP-3 antibody, (F) with anti-MASP-2 antibody reacting with MAp19, (G) MBL in fractions from ion-exchange chromatography, and (H) C3 activating capacities of the same fractions (note the C3a′ chain in lanes 4 and 5).

step in generating the C3 convertase, C4bC2b, coincided with the MBL-II complexes (Figure 2A). Also, when fractions from the ion-exchange chromatography were tested the C4 activation could be assigned to the MASP-2 containing fractions (data not shown). The direct activation of C3 was associated with the MBL-I complexes (Figure 2H).

MBL complexes were removed from serum through binding to solid phase anti-MBL. Western blotting analysis showed that all the MBL but only part of the MASP-3 was recovered from the solid phase. The residual MASP-3 in the supernatant could be recovered by adding further MBL and repeating the affinity step (data not shown).

Activity of MASP-3

We examined the functional activity of MASP-3 by incubating rMASP-3 with MBL complexes. This revealed a pronounced inhibitory activity of rMASP-3 on the activation of C4 by MBL complexes purified from plasma (Figure 3A). The activity of rMBL-rMASP-2 complexes (Vorup-Jensen et al., 2000) was similarly inhibited by rMASP-3 (data not shown). The controlling activity of MASP-3 appears to be mediated through competitive inhibition of association of MASP-2 to MBL (compare lane 1 with 3 and 4 with 6, Figure 3B). At the same time, a decrease could be seen in the activation of the MASP-2 still associated with MBL (observe the 52 k M_r band representing the A chain of activated MASP-2, lanes 7 and 9, Figure 3B). The band at 75 k M_r represents the proenzyme form of MASP-2. There was no indication of enzymatic cleavage of MASP-2 by MASP-3.

The Primary Structure of MASP-3 and the Organization of the Gene

Further sequencing of MASP-3-derived peptides yielded amino acid sequences, which were used to design and synthesize degenerated oligonucleotides. These were used for PCR amplification, yielding a 174 base

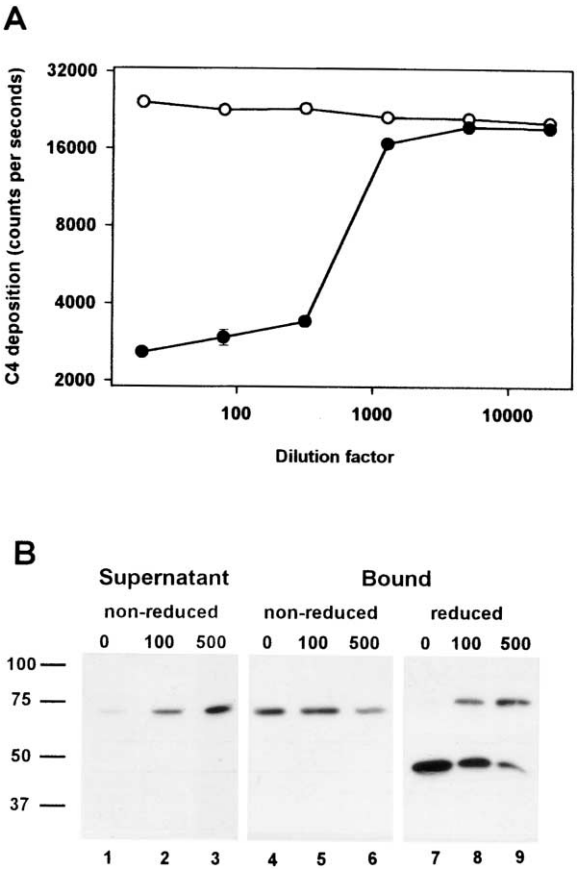

Figure 3. MASP-3 Inhibits the MBL Pathway

(A) Dilutions of rMASP-3 (filled circles) or control (open circles) were incubated with plasma-derived MBL complexes in mannan-coated wells, C4 was added, incubated at 37°C, and bound C4 fragments were quantified with Eu-labeled anti-C4 antibody. Activity (%) was read from a standard curve based on dilutions of MBL complexes. rMASP-3 was in this experiment used in the form of culture supernatants of transfected cells with supernatant of sham-transfected cells as control. The same results were obtained with rMASP-3 purified by ion-exchange chromatography.

(B) Western blotting analysis of the inhibitory activity of MASP-3 on MASP-2. MBL (100 ng/ml) (MASP free plasma derived MBL) (Vorup-Jensen et al., 2000) and rMASP-2 (10 ng/ml) were mixed and incubated for 2 hr at 37°C without rMASP-3 or with 100 or 500 ng/ml rMASP-3 in mannan-coated wells. Supernatant was collected. The wells were washed and bound material eluted with sample buffer. Supernatant and eluate were subjected to SDS-PAGE at reducing or nonreducing conditions, and blotted and developed with anti-MASP-2 antibody. The amount of MASP-2 in the supernatant increases with MASP-3 addition. In parallel the amount of MBL bound MASP-2 decreases. The activation of the bound MASP-2 decreased upon MASP-3 addition as seen in the samples run at reducing conditions (the 52 k M_r band represents the A chain of activated MASP-2).

nucleotide fragment from liver cDNA. The deduced amino acid sequence (Figure 4A) classified the protein as a protease homologous to the B chains of MASP-1, MASP-2, C1r, and C1s. The 174 bp DNA was found to be included in the genomic sequence AC007920. This 230 kb sequence of unsorted fragments was found to contain the entire MASP-3 B chain sequence as judged by comparison with the B chains of MASP-1, MASP-2, C1r, and C1s. In addition, it contained the sequence for the 10 exons encoding the MASP-1 A chain and the 6 exons encoding the MASP-1 B chain. The relevant fragments were sorted on the basis of the published genome sequence of MASP-1 (Endo et al., 1998), yielding the genomic structure shown schematically in Figure 4C. The exon for the MASP-3 B chain is located between the 10 exons encoding the MASP-1 A chain and the 6 exons encoding the MASP-1 B chain.

Primers were synthesized corresponding to the 5′ and 3′ ends of the MASP-3 B chain and used for PCR amplifications from genomic DNA and liver cDNA. Both reactions yielded DNA fragments, which were cloned and sequenced and found to agree 100% with the sequence for the B chain in the database. Thus, in contrast to the MASP-1 B chain but like the B chains of MASP-2, C1r, and C1s, the MASP-3 B chain is encoded by a single exon.

Cloning of MASP-3 cDNA from a human liver library revealed a transcription product composed of a common MASP-1/3 A chain and a unique MASP-3 B chain. The largest clone, encoding full-length MASP-3 (pMASP-3;4.1) comprises 3595 bp, starting with a 5′ untranslated region of 90 bp, followed by an open reading frame (ORF) of 2184 bp and a 3′ untranslated region of 1321 bp, and ending with a poly-A tail. The nucleotide sequence of pMASP-3;4.1 has been deposited in GenBank (accession number AF284421). The amino acid sequences of the sequenced peptides were identified in the sequence deduced from the clone (Figure 4A). The ORF encodes a polypeptide chain of 728 amino acids, including a signal peptide of 19 residues. Three N-glycosylation sites are found in the B chain and four in the A chain. Omitting the signal peptide, the calculated mass is 81,873 as compared with M_r105 k M_r observed on SDS-PAGE. The calculated isoelectric point is 5.02, and the molar extinction coefficient at 280 nm is 121,610 (absorbance of 1 g/liter = 1.49). The alternative splicing site was shown to be situated immediately after exon 10. The open reading frame of the B chain starts with a 42 bp untranslated sequence followed by the codons for the 14 residue link region. This link region precedes the activation site where the split between the A and B chains takes place (Figure 4C).

The last domain of the A chain, CCP2, is encoded by exons 9 and 10. Exon 10 is followed by an intron and the exon encoding the MASP-3 B chain. This structure was confirmed by PCR on human liver cDNA using a primer pair corresponding to a sequence from exon 9 of the MASP-1 A chain and a sequence from the MASP-3 B chain. Antibody raised against a peptide representing the 20 N-terminal residues of the MASP-1 A chain recognized MASP-3 on Western blots as identified in parallel by the anti-MASP-3 B chain antibody and by an antibody raised against a peptide representing the MASP-3 link region (data not shown), thus identifying the MASP-3 protein as a product arising from alternative splicing.

Evolutionary Aspects

Database searches revealed homology of the MASP-3 B chain with sequences logged for shark and carp MASP (Endo et al., 1998; Figure 4B). The sequence identities are 65% and 62%, respectively, whereas those between human MASP-3 B chain and human MASP-1 and

MASP-2 B chains are only 37% and 38%, respectively. Lamprey MASP shares a number of structural features with shark and carp MASP (Endo et al., 1998).

Two sequences logged for porcine DNA together match the 182 C-terminal residues of the human MASP-3 B chain. A 95% amino acid sequence identity between the two proteins was revealed, with only three differences within the first 170 residues (Figure 4B). The nucleotide identity was 89%.

Discussion

We have identified and characterized a MBL-associated serine protease, MASP-3. Thus, the family of MASP-like serine proteases now encompasses five members: MASP-1, MASP-2, MASP-3, C1r, and C1s. All these proteins show identical domain organization, even though the overall amino acid sequence identity is only ~40%. The family name is suggested since MASP-1 phylogenetically stands out as the likely earliest of these proteins due to the chymotrypsin-like nature of its serine protease domain. As is the case for chymotrypsin, the protease domain of MASP-1 is encoded by several exons, and the structure around the active site histidine is stabilized by a disulphide bridge (the histidine loop). The active site serine residue in MASP-1, like in chymotrypsin, is encoded by a TCN codon (N = any nucleotide). These features are also found for MASPs in protochordates (MASPa and MASPb of the ascidian *Halocynthia roretzi*; Endo et al., 1998), and also in the other serine proteases of the complement system, factor D, factor B, C2, and factor I. In contrast, for MASP-2, MASP-3, C1r, and C1s the serine protease domain is encoded by a single exon, there is no histidine loop, and the active site serine is encoded by AGY (Y = C or T). This is the case for mammals as well as for MASPs in the frog, carp, shark, and lamprey. The split exon structure of the gene and the codon usage are features that have no structural implications, but they serve as useful markers for phylogenetic considerations. The histidine loop could have functional implications. A phylogenetic tree suggests that the AGY type diverged from the TCN type before the emergence of primitive vertebrates (Endo et al., 1998). While C1r and C1s have been found only in advanced vertebrates, the early appearance of MASPs suggests the premordial nature of MASPs and the MBL pathway of complement activation. The maintenance of these proteins in all higher animals points to importance of this system for the preservation of an optimal innate immune defense.

MASP-3 is unusually conserved with pig and rat (W. Schwable, personal communication) B chains both showing more than 90% sequence identity to the human B chain, and an identity of 62% and 65% to carp and shark MASP-3, respectively. Strikingly, in a consecutive sequence of 170 residues there are only three differences between human and pig MASP-3 B chain. In comparison, the B chains of the other four MASP-like serine proteases show a more usual degree of conservation of about 80% between mammals (e.g., human and rat) and 30% to 40% between mammalian and lower vertebrate homologs. This is the same degree of conservation seen between the different MASP-like serine proteases within a species (Figure 5). The coding region of the MASP-1/3 gene comprises from the 5′ end: 10 exons encoding the common MASP-1/3 A chain, followed by a single exon encoding the MASP-3 link region and protease domain, and next the 6 exons encoding the MASP-1 link region and protease domain. There are poly-A regions following the exons encoding the protease domains of both MASP-3 and MASP-1. The MASP-3 B chain presumably arose through partial gene duplication of the MASP-1 gene. After modifications including loss of the introns and loss of the cysteines forming the histidine loop, the locus encompassing the MASP-1/3 A chain and the MASP-3 B chain proceeded to duplicate and diversify to generate the group of AGY MASP-like serine proteases.

Another aspect of this study is the demonstration of the selective association of MASP-3 with MBLs of a restricted degree of oligomerization (Figure 2). Likewise, the other MASPs and MAp19 show a restricted distribution among MBL oligomers. The restricted distribution of the MASPs was found to be reflected in the biological activities of the complexes, with the smaller oligomers containing MASP-1 and MAp19 showing direct activation of C3. Higher oligomers containing MASP-2 activated C4 and C2 to generate the C3 convertase C4bC2b. This agrees with previous observations on the activity of isolated MASP-1 (Matsushita and Fujita, 1995a) and MASP-2 (Thiel et al., 1997; Matsushita et al., 2000). It has also recently been shown that complexes composed only of rMASP-2 and MBL can activate C4 (Vorup-Jensen et al., 2000). MASP-3 was largely found with the MASP-2 containing complexes. Superior separation procedures are needed to clearly resolve the different complexes for determining their function and composition, and to establish the degree of oligomerization of the MBL. It is noteworthy that the size separations at physiological conditions were directly reflected in the mobilities of the MBL oligomers on nonreduced SDS-PAGE, suggesting that the oligomerization is defined by disulphide bridges and not by noncovalent interactions. The presence of distinct MBL oligomers agrees with previous findings (Lu et al., 1990; Lipscombe et al., 1995; Yokota et al., 1995). It was unexpected to find that the MASPs are differentially associated with distinct MBL oligomers.

After storage at 4°C of the different forms of MBL complexes separated by chromatography, we do not see any change in the oligomerization as analyzed by SDS-PAGE, suggesting that the oligomers once formed are stable. It is remarkable that the protein generating machinery is capable of producing these distinct forms from a single polypeptide chain.

The current study of oligomers was directed at the wild-type form of MBL (the A/A allotype) only. In MBL from pooled plasmas one will observe also a very minor staining on the Western blots of molecular forms at around 100 k M_r, representing MBL from individuals with mutations in the MBL gene (Lipscombe et al., 1995). These mutated forms previously have been found not to bind MASP-1 and not to activate complement (Matsushita et al., 1995b).

To understand the biology of the MASPs, it is important to realize that only a proportion of these proteases are associated with MBL in serum, as has been demon-

A

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huMASP-3        RIIGGRNAEPGLFPWQALIVVEDTSRVPNDKWFGSGALLSASWILTAAHVLRSQR----- 55
huMASP-1        RIFNGRPAQKGTTPWIAMLSHLNGQP------FCGGSLLGSSWIVTAAHCLHQSLDPGDP 54
huMASP-2        RIYGGQKAKPGDFPWQVLGGTTAAG---------ALLILYDNWVLTAAHAVYEQK----- 46
                ** .*: *: *  ** .:              .  :*  .*::**** : ..

huMASP-3        --RDTTVIPVSKEHVTVYLGLHDVR-DKSGAVNSSAARVVLHPDFNIQNYNHDIALVQLQ 112
huMASP-1        TLRDSDLLSPSD--FKIILGKHWRLRSDENEQHLGVKHTTLHPQYDPNTFENDVALVELL 112
huMASP-2        --HDASALDIR-------MGTLKRL-SPHYTQAWSEAVFIHEGYTHDAGFDNDIALIKLN 96
                  :*:  :          :*      .       .      .   .   :::*:**::*

huMASP-3        EPVPLGPHVMPVCLPRLEPE-GPAPHMLGLVAGWGISNPNVTVDEIISSGTRTLSDVLQY 171
huMASP-1        ESPVLNAFVMPICLPEGPQQ----EGAMVIVSGWGKQFLQR------------FPETLME 156
huMASP-2        NKVVINSNITPICLPRKEAESFMRTDDIGTASGWGLTQR--------G----FLARNLMY 144
                :   :.. : *:***.   :       :  .:***                 :.  *

huMASP-3        VKLPVVPHAECKTSYESRSG-NYSVTENMFCAGYYEGGKDTCLGDSGGAFVIFDDLSQRW 230
huMASP-1        IEIPIVDHSTCQKAYAPLKK---KVTRDMICAGEKEGGKDACAGDSGGPMVTLNRERGQW 213
huMASP-2        VDIPIVDHQKCTAAYEKPPYPRGSVTANMLCAGLESGGKDSCRGDSGGALVFLDSETERW 204
                :.:*:* *  *  :*        .** :*:***  .****:* *****.:* ::    :*

huMASP-3        VVQGLVSWGGPEECGSKQVYGVYTKVSNYVDWVWEQMGLPQSVVEPQVER            280
huMASP-1        YLVGTVSWG--DDCGKKDRYGVYSYIHHNKDWIQRVTGVRN                      252
huMASP-2        FVGGIVSWGSMN-CGEAGQYGVYTKVINYIPWIENIISDF                       243
                 : * ****  : **.   ****: : :   *: .  .
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B

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huMASP-3        RIIGGRNAEPGLFPWQALIVVEDTSRVPNDKWFGSGALLSASWILTAAHVLRSQRRDTTV 60
pig             ------------------------------------------------------------
Shark           RIIGGRTAAPGFFPWQLLIVVEDVSRVPKDKWFGSGALLSRTWVLTAAHVLRSQRRD-TI 59
Carp            RIVGGRTASPGLFPWQVLLSVEDVSRVPEDRWFGSGALLSSTWVLTAAHVLRSHRRDFSV 60
                **:***.* **:**** *: ***.****:*:********* :*:*********:*** ::

huMASP-3        IPVSKEHVTVYLGLHDVRDKSGAVNSSAARVVLHPDFNIQNYNHDIALVQLQEPVPLGPH 120
Pig             ----------------------------------------IQNYNHDIALVQLQEPVPLGPH
Shark           TLVPSEYVTIYLGLHDVRQKEAAAKRTVEKIILHKAFDPRTYNNDIALVKMKDKVSMNVF 119
Carp            VPVASEHIRVHLGLTDIRDKHLATNRSVAKVILHPQFDPQNYNNDIALIKLSQEVVLSAL 120
                  *..*:: ::*** *:*:*  *.: :. :::**  *: :.**:****:::.: * :.

huMASP-3        VMPVCLPRLEPEG----PAPHMLGLVAGWGISNPNVTVDEIISS-GTRTLSDVLQYVKLP 175
Pig             VMPVCLPRPEPEG----PAPHMLGLVAGWGISNPGVTVDEIISS-GTRTLSDVLQYVKLP 30
Shark           VMPLCLPSLHQEME--EPQPNTLGLVAGWGITNPNLTLDDASGS-DQATLSNILQYVKLP 176
Carp            IQPVCLPRPGVKGHTLMPLPNTLGIVAGWGINTANTSASTSGLTSDLGTVSELLQYVKLP 180
                : *:***    :     * *: **:******.... : .    : .  *:*::*******

huMASP-3        VVPHAECKTSYESRSGNYSVTENMFCAGYYEGGKDTCLGDSGGAFVIFDDLSQRWVVQGL 235
Pig             VVPHAECKTSYESRSGNYSVTENMFCAGYYEGGKDTCLGDSGGAFVILDDLSQRWVAQGL 90
Shark           VTLQAECKSSYESRSDSYNVTDNMFCAGFYEGGKDTCLGDSGGAFITYDSSTQSWVAQGL 236
Carp            IVPQDECEASYASRSVNYNITSNMFCAGFYEGGQDTCLGDSGGAFVTQDARSGRWVAQGL 240
                :. : **::** *** .*.:*.******:****:***********:  *  :  **.***

huMASP-3        VSWGGPEECGSKQVYGVYTKVSNYVDWVWEQMGLPQSVVEPQVER                  280
Pig             VSWGGPEECGSKQVYGVYTKVSNYVDWVWEQMGSPPGLGELQVER                  135
Shark           VSWGGPEKCGSKRVYGVYTKISKYARWLADKMSNSSD                          273
Carp            VSWGGPEECGSQRVYGVYTRVANYIHWLHRHMDGEEVAKV                       280
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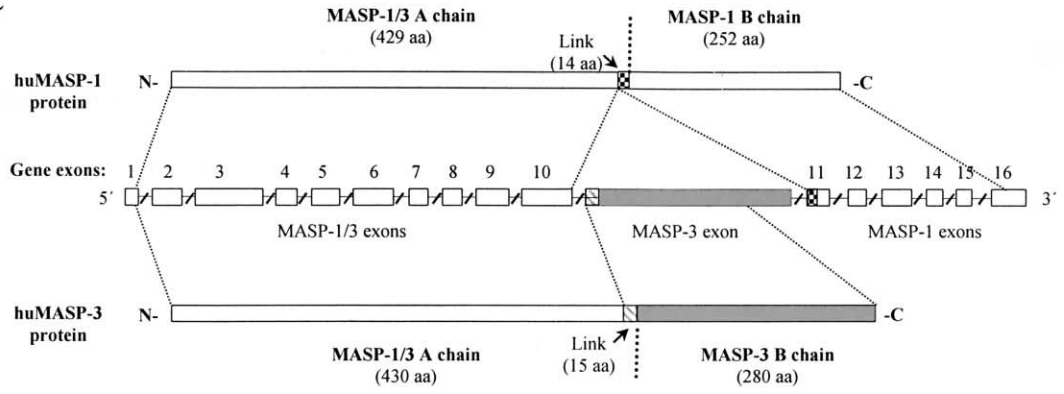
C



	hu MASP-1	hu MASP-2	mo MASP-1	mo MASP-2	xe MASP-1	xe MASP-2	ca MASP-3	sh MASP-3	la MASP	as MASPa	as MASPb	hu C1r	hu C1s
huMASP-3	33	37	33	35	35	38	**62**	**65**	38	28	29	35	35
huMASP-1		42	**82**	32	55	35	33	33	33	30	29	29	29
huMASP-2			33	**78**	32	50	35	34	39	34	27	38	37
moMASP-1				31	54	32	34	33	32	29	29	32	29
moMASP-2					33	49	36	36	38	36	27	37	37
xeMASP-1						35	32	35	34	31	28	32	31
xeMASP-2							37	35	38	33	28	37	40
caMASP-3								**62**	39	27	29	32	33
shMASP-3									38	29	27	29	34
laMASP										34	27	35	40
asMASPa											29	30	34
asMASPb												25	29
huC1r													41

Figure 5. Identities between B Chains of the MASP-like Serine Proteases

Percentage identity was calculated based on the alignment of the amino acid sequences. The sequences used for the alignments have the following accession numbers: laMASP, AB009075; xeMASP-1, D83276; xeMASP-2, AB009072; huC1r, M14058; huC1s, CAA29817; asMASPa, BAA19762; asMASPb, BAA19763; moMASP-1, NP 032581; and moMASP-2, BAA34674. The following abbreviations were used: hu, human; mo, mouse; xe, *Xenopus*; la, lamprey; sh, shark; as, ascidian; and ca, carp.

strated for MASP-1 and MASP-2 (Terai et al., 1997; Thiel et al., 2000). By depleting serum of MBL complexes and analyzing for residual MASP-3 by Western blotting, we found the same to be true for this protein.

The functional activity of MASP-3 was assessed in experiments on opsonization, i.e., MBL/MASP-2 mediated C4b deposition on mannan-coated surfaces. In such assays, MASP-3 demonstrated inhibition of MASP-2 effected by a combination of competitive inhibition of MASP-2 association with MBL and inhibition of activation of the MBL-associated MASP-2. The initiating complex of the classical complement activation pathway, C1, is a single entity comprised of the hexameric C1q in complex with two C1r and two C1s molecules. It was initially expected that the MBL complex could be modeled on the C1 structure, but the present report emphasizes structural and functional differences between the two complexes.

Limitation of infection upon exposure to microbes is of utmost importance for the survival of the individual. Thus, several systems have evolved for this purpose. Cellular components, e.g., toll-like receptors (Medzhitov and Janeway, 2000) and receptors for "non-self" carbohydrate compositions (Hoffmann et al., 1999), as well as humoral factors, e.g., MBL, C-reactive protein (Du Clos, 2000) and natural antibodies (Ochsenbein and Zinkernagel, 2000), are involved. Upon binding to their ligands, these molecules mediate signals to other components of the body's defense system, establishing a range of immediate antimicrobial defense functions but also influencing the selection and the modes of the following adaptive immune responses (Medzhitov and Janeway, 1997). The new protein MASP-3, characterized in the present paper, as well as the other MASPs have the potential to perform such signaling functions when MBL binds to carbohydrate structures.

Experimental Procedures

Amino Acid Sequencing

A lectin preparation purified from plasma (Thiel et al., 1997) was subjected to SDS-PAGE, transferred to a PVDF membrane, and stained with Coomassie brilliant blue. The 42 k M_r band was cut out and subjected to sequencing on an applied biosystems protein sequencer. Peptides were prepared by tryptic digestion of the 42 k M_r band from a Coomassie-Blue-stained SDS-PAGE gel, fractionated by reverse phase chromatography, and the peptides in the major peaks were sequenced.

Purification and Physical Characterization of MBL Complexes

MBL complexes were purified from normal human plasma by affinity chromatography on mannan-Sepharose in the presence of enzyme

Figure 4. The Sequence and the Genomic Organization of MASP-3

(A) Deduced amino acid sequence of the MASP-3 B chain. The sequence is aligned with those of human MASP-1 (NM001879) and human MASP-2 (Y09926) B chains.

(B) Alignment of human MASP-3 with shark (AB009074) and carp (AB009073) MASP-3 B chains and a partial pig MASP-3 sequence (a combination of AW414970 and BE030550). (*) identical residues, (:) conserved substitutions, (.) semiconserved substitutions. The alignments were made with BLOSUM 62. Aligned cysteines are boxed. The cysteines in the histidine loop of MASP-1 are shaded. The three potential N-glycosylation sites of MASP-3 are in bold. The three arrows indicate the active site residues of the serine proteases. Sequences identified by protein sequencing are underlined.

(C) Genomic organization of the exons (drawn to scale) encoding MASP-1 and MASP-3 and comparison of the derived proteins.

inhibitors and were eluted with mannose-containing buffer (Matsushita et al., 2000). Sucrose gradient centrifugation was performed by applying 100 μl MBL complex or 30 μl serum samples diluted with 70 μl Tris-buffered saline (TBS) onto 11 ml sucrose gradients (10%–30%) in TBS containing 5 mM $CaCl_2$ and 50 μg/ml human serum albumin and centrifuging at 35,000 rpm at 4°C for 24 hr in a Beckman L8-M centrifuge with a Sorval TST 41.14 rotor. Fractions of 0.3 ml were collected and the positions of IgG, IgM, and MBL sedimentation peaks were determined by time-resolved immunofluorometric assays (TRIFMAs)(Thiel et al., 2000). For ion-exchange chromatography, MBL complexes were dialyzed against 20 mM Tris/HCl (pH 7.8), containing 50 mM NaCl and 10 mM $CaCl_2$, and fractionated on a 1 ml Mono Q column (Amersham-Pharmacia, Uppsala, Sweden) with an NaCl gradient to 0.5 M. Fractions of 0.5 ml were collected and analyzed for MBL by TRIFMA. Fractions were also analyzed by SDS-PAGE Western blotting against anti-MBL (Statens Serum Institut, Copenhagen, Denmark), anti-MASP-1 (Thiel et al., 1997), anti-MASP-2 (Thiel et al., 2000), or anti-MASP-3 antibodies. Anti-MASP-3 antibody was raised against a peptide representing the first 19 amino acid residues of the 42 k M_r A chain by the method described (Thiel et al., 1997). The blots were treated with horseradish peroxidase-labeled secondary antibody (DAKO, Glostrup, Denmark) followed by enhanced chemiluminescence reagent (Pierce Chemicals, Rockville, IL) and exposure to X-ray film. Markers for calculating M_rs were from BioRad, Richmond, CA ("Precision Standards"), α2M and IgM from Sigma, St. Louis, MO.

Biological Activity of MBL Complexes

The ability of the MBL complexes in various fractions to mediate activation of C3 (Matsushita and Fujita, 1995a) was assessed by incubating 10 μl samples of fractions from ion-exchange chromatography with 10 μl (2 μg) purified C3 (Dodds, 1986) at 37°C for 1 hr before analyzing the digest by SDS-PAGE under reducing conditions and staining with Coomassie brilliant blue. Activation of C4 was assessed by incubating samples at 4°C in microtiter wells coated with mannan, followed by incubation at 37°C with purified C4 (Dodds, 1986) and development with Eu-labeled monoclonal anti-C4 antibody (Thiel et al., 2000).

Cloning of MASP-3 cDNA

PCR was performed on human liver cDNA (Clontech, Palo Alto, CA) using degenerated sense and antisense primers derived from the amino acid sequences WQALIVVE and EHVTVYL, respectively. The PCR was carried out with annealing at 48°C for 30 cycles using the long expand PCR system from Boehringer Mannheim, Mannheim, Germany. The resulting 174-bp PCR product was cloned into an *E. coli* plasmid (2.1-TOPO, InVitrogen, Groningen, The Netherlands) and the nucleotide sequence of the insert determined. By BLAST search of the GenBank database, this sequence identified a genomic fragment of 230 kb made up by random fragments (AC007917). Specific primers were used to obtain two cDNA clones (pMASP-3; 4.1 and pMASP-3; 3.0) in the pEAK8 vector (Pangene, Mountain View, CA). The inserts contained an open reading frame of 2163 bp encoding full-length MASP-3.

Recombinant MASP-3

Synthesis of rMASP-3 was accomplished by a procedure reported earlier for rMASP-2 (Vorup-Jensen et al., 2000). In brief, human embryonic kidney cells expressing the Epstein-Barr nuclear antigen (HEK 293EBNA, InVitrogen) were transfected with the pEAK8/pMASP-3; 4.1 construct and cultured in RPMI-1640 supplemented with insulin, transferrin, and selenium (Life Technologies, Paisly, UK). The culture supernatant was harvested after 6 days. A control was prepared by incubating the HEK 293EBNA cells with calcium phosphate precipitate without the construct. In some experiments, the rMASP-3 was purified from the culture supernatant by ion-exchange chromatography on a Mono Q column. Fractions containing rMASP-3 were pooled and further purified by size permeation chromatography. On analysis by SDS-PAGE and protein staining with silver ions, the rMASP-3 appeared at least 95% pure after this procedure.

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References

Carroll, M.C. (2000). The role of complement in B cell activation and tolerance. Adv. Immunol. *74*, 61–88.

Dempsey, P.W., Allison, M.E.D., Akkaraju, S., Goodnow, C.C., and Fearon, D.T. (1996). C3d of complement as a molecular adjuvant: bridging innate and acquired immunity. Science *271*, 348–350.

Dodds, A.W. (1986). Small scale preparation of complement components C3 and C4. Meth. Enzymol. *223*, 46–61.

Du Clos, T.W. (2000). Function of C-reactive protein. Ann. Med. *32*, 274–278.

Endo, Y., Takahashi, M., Nakao, M., Saiga, H., Sekine, H., Matsushita, M., Nonaka, M., and Fujita, T. (1998). Two lineages of mannose-binding lectin-associated serine protease (MASP) in vertebrates. J. Immunol. *161*, 4924–4930.

Fearon, D.T., and Locksley, R.M. (1996). The instructive role of innate immunity in the acquired immune response. Science *272*, 50–53.

Fearon, D.T., and Carroll, M.C. (2000). Regulation of B lymphocyte responses to foreign and self-antigens by the CD19/CD21 complex. Annu. Rev. Immunol. *18*, 393–422.

Fischer, M.B., Ma, M., Goerg, S., Zhou, X., Xia, J., Finco, O., Han, S., Kelsoe, G., Howard, R.G., Rothstein, T.L., et al. (1996). Regulation of the B cell response to T-dependent antigens by classical pathway complement. J. Immunol. *157*, 549–556.

Garred, P., Pressler, T., Madsen, H.O., Frederiksen, B., Svejgaard, A., Hoiby, N., Schwartz, M., and Koch, C. (1999). Association of mannose-binding lectin gene heterogeneity with severity of lung disease and survival in cystic fibrosis. J. Clin. Invest. *104*, 431–437.

Ghiran, I., Barbashov, S.F., Klickstein, L.B., Tas, S.W., Jensenius, J.C., and Nicholson-Weller, A. (2000). Complement receptor 1/CD35 is a receptor for mannan-binding lectin. J. Exp. Med. *192*, 1797–1808.

Hoffmann, J.A., Kafatos, F.C., Janeway, C.A., and Ezekowitz, R.A.B. (1999). Phylogenetic perspectives in innate immunity. Science *284*, 1313–1318.

Ikeda, K., Sannoh, T., Kawasaki, N., Kawasaki, T., and Yamashina, I. (1987). Serum lectin with known structure activates complement through the classical pathway. J. Biol. Chem. *262*, 7451–7454.

Janeway, C.A., Jr. (1989). Approaching the asymptote? Evolution and revolution in immunology. Cold Spring Harb. Symp. Quant. Biol. *54*, 1–13.

Ji, Y.-H., Fujita, T., Hatsuse, H., Takahashi, A., Matsushita, M., and Kawakami, M. (1993). Activation of the C4 and C2 components of complement by a proteinase in serum bactericidal factor, Ra reactive factor. J. Immunol. *150*, 571–578.

Kuhlman, M., Joiner, K., and Ezekowitz, R.A.B. (1989). The human mannose-binding protein functions as an opsonin. J. Exp. Med. *169*, 1733–1745.

Law, S.K.A. and Reid, K.B.M. (1995). Complement (Oxford: IRL Press).

Lipscombe, R.J., Sumiya, M., Summerfield, J.A., and Turner, M.W. (1995). Distinct physiochemical characteristics of human mannose binding protein expressed by individuals of differing genotypes. Immunology *85*, 660–667.

Lipscombe, R.J., Beatty, D.W., Ganczakowski, M., Goddard, E.A., Jenkins, T., Lau, Y.L., Spurdle, A.B., Sumiya, M., Summerfield, J.A., and Turner, M.W. (1996). Mutations in the human mannose-binding protein gene: frequencies in several population groups. Eur. J. Hum. Genet. *4*, 13–19.

Lu, J., Thiel, S., Wiedemann, H., Timpl, R., and Reid, K.B.M. (1990).

Binding of the pentamer/hexamer forms of mannan-binding protein to zymosan activates the proenzyme C1r2C1s2 complex, of the classical pathway of complement, without involvement of C1q. J. Immunol. *144*, 2287–2294.

Madsen, H.O., Garred, P., Thiel, S., Kurtzhals, J.A., Lamm, L.U., Ryder, L.P., and Svejgaard, A. (1995). Interplay between promoter and structural gene variants control basal serum level of mannan-binding protein. J. Immunol. *155*, 3013–3020.

Matsushita, M., and Fujita, T. (1992). Activation of the classical complement pathway by mannose-binding protein in association with a novel C1s-like serine protease. J. Exp. Med. *176*, 1497–1502.

Matsushita, M., and Fujita, T. (1995a). Cleavage of the third component of complement (C3) by mannose-binding protein-associated serine protease (MASP) with subsequent complement activation. Immunobiol. *194*, 443–451.

Matsushita, M., Ezekowitz, R.A.B., and Fujita, T. (1995b). The Gly-54→Asp allelic form of human mannose-binding protein (MBP) fails to bind MBP-associated serine protease. Biochem. J. *311*, 1021–1023.

Matsushita, M., Thiel, S., Jensenius, J.C., Terai, I., and Fujita, T. (2000). Proteolytic activities of two types of mannose-binding lectin-associated serine protease. J. Immunol. *165*, 2637–2642.

Medzhitov, R., and Janeway, C. (2000). Innate immune recognition: mechanisms and pathways. Immunol. Rev. *173*, 89–97.

Ochsenbein, A.F., and Zinkernagel, R.M. (2000). Natural antibodies and complement link innate and acquired immunity. Immunol. Today *21*, 624–630.

Pepys, M.B. (1974). Role of complement in induction of antibody production in vivo: effect of cobra venom factor and other C3-reactive agents on thymus-dependent and thymus-independent antibody responses. J. Exp. Med. *140*, 126–145.

Selander, B., Weintraub, A., Holmstrom, E., Sturfelt, G., Truedsson, L., Martensson, U., Jensenius, J.C., and Sjoholm, A.G. (1999). Low concentrations of immunoglobulin G antibodies to Salmonella serogroup C in C2 deficiency: Suggestion of a mannan-binding lectin pathway-dependent mechanism. Scand. J. Immunol. *50*, 555–561.

Stover, C.M., Thiel, S., Thelen, M., Lynch, N.J., Vorup-Jensen, T., Jensenius, J.C., and Schwaeble, W. (1999). Two constituents of the initiation complex of the mannan-binding lectin activation pathway of complement are encoded by a single structural gene. J. Immunol. *162*, 3481–3490.

Summerfield, J.A., Sumiya, M., Levin, M., and Turner, M.W. (1997). Association of mutations in mannose-binding protein gene with childhood infections in consecutive hospital series. Brit. Med. J. *314*, 1229–1232.

Super, M., Thiel, S., Lu, J., Levinsky, R.J., and Turner, M.W. (1989). Association of low levels of mannan-binding protein with a common defect of opsonisation. Lancet *2*, 1236–1239.

Takahashi, M., Endo, Y., Fujita, T., and Matsushita, M. (1999). A truncated form of mannose-binding lectin-associated serine protease (MASP)-2 expressed by alternative polyadenylation is a component of the lectin complement pathway. Int. Immunol. *11*, 859–863.

Terai, I., Kobayashi, K., Matsushita, M., and Fujita, T. (1997). Human serum mannose-binding lectin (MBL)-associated serine protease-1 (MASP-1): determination of levels in body fluids and identification of two forms in serum. Clin. Exp. Immunol. *110*, 317–323.

Thiel, S., Vorup-Jensen, T., Stover, C.M., Schwaeble, W., Laursen, S.B., Poulsen, K., Willis, A.C., Eggleton, P., Hansen, S., Holmskov, U., Reid, K.B.M., and Jensenius, J.C. (1997). A second serine protease associated with mannan-binding lectin that activates complement. Nature, *386*, 506–510.

Thiel, S., Petersen, S.V., Vorup-Jensen, T., Matsushita, M., Fujita, T., Stover, C.M., Schwaeble, W., and Jensenius, J.C. (2000). Interaction of C1q and mannan-binding lectin (MBL) with C1r, C1s, MBL-associated serine protease 1 and 2 and MAp19. J. Immunol. *165*, 878–887.

Turner, M.W. (1996). Mannose-binding lectin: the pluripotent molecule of the innate immune system. Immunol. Today *17*, 532–540.

Turner, M.W., and Hamvas, R.M. (2000). Mannose-binding lectin: structure, function, genetics and disease associations. Rev. Immunogenet. *2*, 305–322.

Vorup-Jensen, T., Petersen, S.V., Hansen, A.G., Poulsen, K., Schwaeble, W., Sim, R.B., Reid, K.B.M., Davis, S.J., Thiel, S., and Jensenius, J.C. (2000). Distinct pathways of mannan-binding lectin (MBL)- and C1-complex autoactivation revealed by reconstitution of MBL with recombinant MBL-associated serine protease-2. J. Immunol. *165*, 2093–2100.

Walport, M.J. (2001a). Complement. N. Engl. J. Med. *344*, 1058–1066.

Walport, M.J. (2001b). Complement. N. Engl. J. Med. *344*, 1140–1144.

Yokota, Y., Arai, T., and Kawasaki, T. (1995). Oligomeric structures required for complement activation of serum mannan-binding proteins. J. Biochem. *117*, 414–419.

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